Celal Bayar University Journal of Science
Volume 15, Issue 2, 2019, p 175-180
Doi: 10.18466/cbayarfbe.493017

Qualitative Analysis of Alfalfa Seed Methanol Extract by GC-MS and Determination of Antioxidant Properties

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Received: 06 December 2018
Accepted: 11 June 2019
DOI: 10.18466/cbayarfbe.493017

Abstract

Alfalfa plant has a great importance for agriculture in Turkey and throughout the world. Antioxidant activities (DPPH and NO scavenging effects) have been studied from the methanol extraction of the seeds, and organic volatile molecule contents have been analyzed by GC-MS. Physical and chemical analysis of the seed were also carried out to elucidate the structure. As a result of the experiments, a concentration-dependent increase was observed in DPPH and NO scavenging (%) activities from the 0.5, 1 and 2 mg/ml methanol extract concentrations of alfalfa seeds. In GC-MS analysis, it was found that squalene, a pharmacologically active molecule, is present in alfalfa seeds. As a result of chemical and physical analyzes, it was determined that the crude protein content is 33.79% and crude oil is 8.11%. Although alfalfa is widely used in agriculture and as animal bait, this study shows that alfalfa seeds are also pharmacologically crucial for containing rich molecules.

Keywords: Clover, squalene, DPPH, NO, scavenging activity.

1. Introduction

Clover (*Medicago sativa L.*) is a perennial, flowering plant from the pea family that can grow in various climatic conditions. The clover plant known in the world as alfalfa and it is one of the most renowned medicinal plants, with bluish-colored flowers, about one meter in length and with steep green leaves [1]. Alfalfa plants are beneficial for both humans and animals. People use alfalfa's sprouts, sensitive roots and dried leaves (which can be used as a dietary supplement in forms such as tablets, powders and tea), animals use the form of hay and feed. Leaves and stems are rich in minerals, proteins and vitamins. The medicinal uses of this plant are as follows: a restorative tonic, antifungal agent for digestion, as well as a food that promotes milk secretion in nursing mothers. Alfalfa is also used as a long-term ayurvedic and homoeopathic drug in central nervous system disorders. Antioxidant, anti-inflammatory and antidiabetic effects of the plant have been reported [2-4].

Nowadays, alfalfa is the world's largest feed produced legumes as feed. In 2009, it was grown in an area of 30 million hectares worldwide. Of this, 11.9 million hectares were planted in North America, 7 million hectares in South America, 7.12 million hectares in Europe, 2.23 million hectares in Asia, 1.75 million hectares in Africa and Oceania [5]. According to the data of 2016 in Turkey, about 630.000 hectares of alfalfa were planted and 9.5 million tons of dry grasses were produced [6].

Antioxidants are micronutrients that can prevent oxidative damage caused by free radicals. Many medicinal plants are rich in antioxidant compounds [3, 4]. To date, many plants have been studied as natural antioxidant sources and various compounds (many of which are polyphenols) have been isolated from these plants. Low and high molecular weight polyphenolic compounds with antioxidant properties were investigated and proposed against lipid peroxidation. Antioxidants are also widely used to prevent degradation of other oxidizable products such as cosmetics, drugs and plastics [7, 8]. Alfalfa contains essential amino acids such as, valine, leucine, threonine and lysine. Due to these essential amino acids and their amount alfalfa is similar to egg white. Green shoots at the top of the plant contain plenty of chlorophyll and various vitamins. These are C, E, B1, B2, B6, B12, niacin, folic acid, biotin, inositol, choline and β-carotene. It also contains valuable minerals such as Ca, Cu, Fe, Mg, Mn, P, Zn, Si [2]. Recent studies have found lithium of 1.12 mg/g in the alfalfa plant growing in the Oman desert [9]. The lithium element is an emotional state regulator and is known to be used in the treatment of bipolar disorders [10]. In terms of proteins and vitamins alfalfa leaf extract has been recognized by the European Food Safety Authority (EFSA) as a rich dietary supplement. Many studies have shown that alfalfa is rich in biologically active compounds as well as nutritional properties [11].

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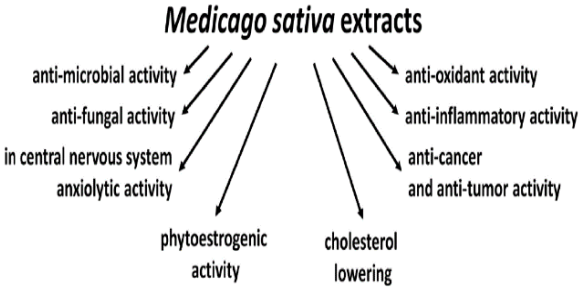

Figure 1. Pharmacological and biological activity of *M. sativa L*. extracts. [12].

Studies to date, the roots, stems and leaves of the alfalfa plant were used as material. There is no sufficient amount of work with the seed part of the plant. In this study we used the seeds of alfalfa plant. Although alfalfa planting is very common in Turkey, properties of its seed has not been widely studied. In this study, methanol extract of alfalfa seeds was prepared and qualitative analysis of gas chromatography / mass spectrometry (GC-MS) was performed from the methanol extract. In this way, organic volatile molecules of the plant seeds were identified. Besides, 1,1 -diphenyl-2-picrylhydrazyl (DPPH) and nitric oxide (NO) free radical scavenging activities were also analyzed in order to elucidate the antioxidant capacity and various chemical and physical analyzes were carried out to reveal the alfalfa seed structure.

2. Material and Method

2.1. Extraction Process

Alfalfa seeds were obtained from a local market in Izmir, Turkey. Before the extraction process, alfalfa seeds were washed in pure water and dried in an oven at 80 °C. For analysis, the seeds were pulverized in high-speed plant mill and prepared for extraction. 2 g of powdered seed samples were extracted in 40 ml of 80% methanol in ultrasonic bath for 30 minutes then left at room temperature. The final extraction concentrate was then adjusted to 50 mg / ml. DPPH and NO scavenging activities in percentage and GC-MS analyzes were determined using this extraction.

2.2 DPPH and NO scavenging Activity

The antioxidant activities of the extracts were tested using the DPPH radical with minor modifications [13]. 2.10^{-4} M DPPH solution was prepared in methanol for this method. 0.5 ml was taken from each sample and on 4 ml of DPPH solution was added. The mixture was shaken and kept at room temperature for 60 minutes in the dark. 80% methanol was used as a blind and samples were read at 517 nm by spectrophotometer (TECAN Infinite M200 microplate reader). The results were calculated according to the following equation.

DPPH scavenging activity %

$= (1\text{-}Abs_{sample} / Abs_{blind}) \times 100$

Nitric oxide scavenging activity was determined according to the following method [14]. Four ml each of the extracts prepared in different concentrations were taken and 1 ml of sodium nitroprusside solution (25 mM) was added to the mixture and incubated for 2 hours at 37 °C. After incubation, 0.5 ml of the solutions were removed and mixed with 0.3 ml of Griess reagent (5% H_3PO_4 in 1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the formed chromophore was read against the blind at 570 nm on the spectrophotometer (TECAN Infinite M200 Microplate reader). The results were calculated

NO scavenging activity % =

$(Abs_{blind} - A_{sample} / Abs_{blind}) \times 100$

2.3. GC / MS Organic Molecule Determination

Volatile molecules in the extract were qualitatively analyzed in electron ionization (EI) mode with Agilent Technology 7890A Gas Chromatography (GC) Mass spectrometer (MS). Chromatographic column Agilent HP-5MS, capillary column (30 m* 0.25 mm, film thickness of 0.25 mm). The furnace temperature was started at 40 ^{0}C, followed by standing for 5 minutes, then at 5 ^{0}C. min^{-1} at 280 ^{0}C. and held for 5 min. Helium gas (99.999%) was used as the carrier gas. The constant flow rate is 1.5 ml min^{-1} and the injector temperature is 250 ^{0}C. The extract was injected in splitless mode with 1 ml. Interpretation of the mass spectrum was performed according to the National Institute of Standards and Technology (NIST) database.

2.4. Other physical and chemical analyzes

Humidity, dry matter, crude protein, crude cellulose, crude oil and ash were analyzed for alfalfa seeds. Kjeldahl method was used in Gerhardt Vapodest 45s device for protein content and Gerhardt fibretherm cellulose device was used for cellulose content. Humidity was determined at 100 ^{o}C in the Memmert etuv. Carbolite ash furnace was used at 600 ^{o}C for ash content. Crude oil content was performed by using Gerhardt soxtherm device.

3. Results and Discussion

3.1. DPPH and NO scavenging Activity

In the study, DPPH scavenging activity in percentage increased by 4.47% at extract concentration of 0.5 mg/ml, and extract concentrations of 1 and 2 mg/ml compared to control group increases of 11.05% and 18.30% respectively. As the extract concentration increased, the DPPH radical scavenging capacity was also increased (Figure 2).

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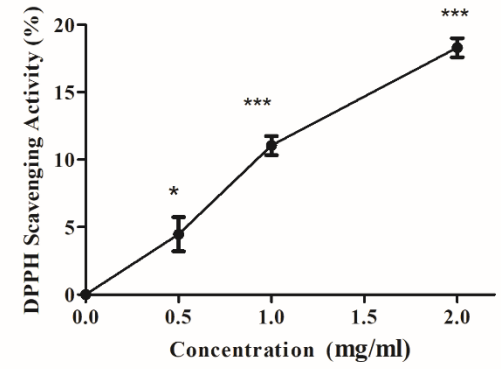

Figure 2. DPPH scavenging activity (%) in alfalfa seed methanol extract (n=3, P<0.05).

Nitric oxide scavenging activities (%) according to the control group; an increase of 37.15% in the amount of 0.5 mg/ml extract, for the concentrations of 1 and 2 mg/ml extracts, NO scavenging levels were increased by 63.67% and 91.82%, respectively. The increase in extract concentration also increased NO inhibition in the medium (Figure 3).

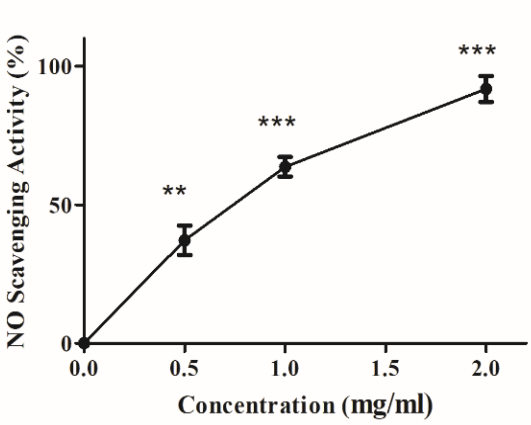

Figure 3. NO scavenging activity (%) in alfalfa seed methanol extract. (n=3, P<0.05).

Alfalfa leaf proteins in a study, a high rate of DPPH radical scavenging activity was also found as the sample concentration increased. Thus, it can be said that alfalfa leaf proteins have a good antioxidant properties and have high DPPH radial scavenging activity [15].

3.2. GC-MS Organic Molecules Determination

Table 1. Results of GC-MS analysis of methanol extract in alfalfa seeds.

Line	Rt	Molecule Name	Similarity %	Molecular Weight (g/mol)
1	5.599	Hexanal	64	100.15
2	6.609	N-Methylcarbamic Acid Ethyl Ester	43	103.12
3	10.036	3-Butenamide	46	85.104
4	12.112	3-hydroxybutyric acid	50	104.105
5	13.743	Erythritol	50	122.120
6	16.501	1-Dodecene	95	168.31
7	20,128	2-Methoxy-4-vinylphenol;	52	150.174
8	21.959	1-Tetradecene	96	196.37
9	22.774	(-)-beta-caryophyllene	96	204.351
10	24.099	Xanthosine	50	284.225
11	24.776	3-Methyl-Thiophene-2-Carboxamide	64	141.191
12	25.045	Phenol, 2,4-bis(1,1-dimethylethyl)	89	206.324
13	26.371	2,6-dimethyl-3-(methoxymethyl)-p-benzoquinone	78	180.200
14	26.796	(-)-Caryophyllene oxide	81	220.350
15	26.841	1-heptadecene	95	238.452
16	27.746	Megastigmatrienone	91	190.281
17	31.243	Trichloroacetic acid,pentadecyl ester	92	373.786
18	31.385	Octadecane	70	254.494
19	32.343	Phytone	83	268.478

20	32.868	1,2-benzenedicarboxylic acid,butyl 2-methylpropyl ester	86	278.343
21	33.413	Decane,3,6-dimethyl	83	170.335
22	**33.957**	**Hexadecanoic acid, methyl ester**	**99**	**270.451**
23	**34.814**	**n-Hexadecanoic acid**	**99**	**256.424**
24	35.182	Estra-1,3,5(10)-trien-17.beta.-ol	87	256.382
25	35.445	Eicosane	86	282.547
26	35.884	Palmitic acid	80	256.424
27	36.979	Patchulane	62	206.366
28	**37.151**	**9,12-octadecadienoic acid methyl ester**	**99**	**294.472**
29	36.360	methyl oleate	60	296.488
30	37.487	4,7-Epoxy-1H-inden-5-ol,1-butyl-2-ethyloctahydro	96	238.366
31	36.473	Oleic Acid	90	282.461
32	38.986	Cyclopentadecanone, 2-hydroxy-	90	240.382
33	39.309	(Z)-9,17-octadecadienal	86	264.446
34	39.461	1-Oxaspiro[2.5]octane, 5,5-dimethyl-4-(3-methyl-1,3-butadienyl)-	93	206.16
35	40.033	9,12-Octadecadienoic acid (Z,Z)-	91	280.445
36	40.128	2-Methyl-Z,Z-3,13-octadecadienol	81	280.496
37	40.759	7-Pentadecyne	93	208.389
38	41.077	12-Methyl-E,E-2,13-octadecadien-1- ol	90	280.276
39	41.636	Hexanoic acid, 2-hexenyl ester,	80	198.302
40	**41.810**	**9-Octadecenamide, (Z)-**	**99**	**281.477**
41	42.226	1,E-11,Z-13-Heptadecatriene	55	234.420
42	42.314	7,11-Hexadecadienal	95	236.393
43	42.878	Diepicedrene-1-oxide	90	220.356
44	43.706	7-Isopropyl-4a-methyloctahydro-2(1H)-naphthalenone	93	208.340
45	44.987	1,2-Benzisothiazole, 3-(hexahydro-1H-azepin-1-yl)-, 1,1-dioxide	93	264.34
46	45.981	Oleic acid, propyl ester	55	324.541
47	46.085	2,5-Furandione,3-dodecyl	62	266.376
48	46.932	13-Tetradecen-1-ol acetate	90	254.414
49	46.980	9,15-Octadecadienoic acid, methyl ester, (Z,Z)	90	294.472
50	48.082	1-Hexacosene	58	364.691
51	**48.747**	**Squalene**	**99**	**410.718**
52	49.120	2-Cyclopropyl-N-(1-cyclopropylethyl)-2-methylcyclopropane-1-carboxamide	83	207.317
53	50.329	2,2-Dimethyl-3-(3,7,16,20-tetramethyl-heneicosa-3,7,11,15,19-pentaenyl)-oxirane	92	412.370
54	**53.407**	**Vitamin E**	**99**	**430.706**

GC-MS analysis of alfalfa seed methanol extracts was carried out qualitatively. The results were compared with the library (NIST) of the device in the database (Table 1). According to the results obtained in alfalfa seed content; hexadecanoic acid methyl ester, n-Hexadecanoic acid, 9,12-octadecadienoic acid methyl

ester, 9-Octadecenamide, (Z)-, squalene, vitamin E, we observed that the molecules exist predominantly.

Squalene is a naturally occurring hydrocarbon. Sterol and hopanoids belong to the wide family of cyclic triterpenes. Squalene is an important intermediate in the biosynthesis of these molecules. Sterols and hopanoids are present in the structure of eukaryotic and prokaryotic organisms [16]. These molecules have critical biological functions such as the sequencing of lipids, membrane regulation [17]. Previous studies have shown that some plants accumulate high squalene. For example, the seeds of the Amaranth plant are now a major source of squalene. [18].

Squalene is a volatile organic compound, but its molecular weight is greater than that of other known volatile molecules such as monoterpenes, sesquiterpenes and green leaf volatiles. There are very few studies currently investigating large molecular weight volatile molecules. Therefore, there are not too many reports of isolation of squalene from plants. [19].

3.3. Other physical and chemical analyzes

According to the results, crude protein was found as 33.79 g and crude oil was 8.11g in 100 g of alfalfa seed (Table 2). In a similar study on alfalfa seed, the seed was made into flour. Total protein, total lipid, ash and crude fiber contents in 100 grams of sample were reported as 34.24, 1.39, 11.65, 21.38 grams, respectively [20].

Table 2. Other physical and chemical analyzes.

Analysis	Amounts (%)
Humidity	8.40
Dry matter	91.6
Crude protein	33.79
Crude cellulose	7.78
Ash	4.21
Crude oil	8.11

4. Conclusion

GC-MS analysis is the first step in understanding the nature of the active substances especially for the medicinal plants. Therefore, in this study, different volatile organic compounds were identified by GC-MS analysis from the methanol extract of alfalfa seeds along with antioxidant activities (DPPH and NO scavenging effects), physical and chemical analysis. This type of work will be useful for a detailed study in the future. Further research related to the pharmacological significance, diversity and detailed biochemistry of alfalfa will be able to add as new information in the traditional medical system.

Author's Contributions

Hafize Dilek Tepe: Drafted and wrote the manuscript, performed the experiment and result analysis.

Ethics

There are no ethical issues after the publication of this manuscript.

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